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Cortactin Interacts with WIP in Regulating Arp2/3 Activation and Membrane Protrusion

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Summary

Background: Modulation of actin cytoskeleton assembly is an integral step in many cellular events. A key regulator of actin polymerization is Arp2/3 complex. Cortactin, an F-actin binding protein that localizes to membrane ruffles, is an activator of Arp2/3 complex.

Results: A yeast two-hybrid screen revealed the interaction of the cortactin Src homology 3 (SH3) domain with a peptide fragment derived from a cDNA encoding a region of WASp-Interacting Protein (WIP). GST-cortactin interacted with WIP in an SH3-dependent manner. The subcellular localization of cortactin and WIP coincided at the cell periphery. WIP increased the efficiency of cortactin-mediated Arp2/3 complex activation of actin polymerization in a concentration-dependent manner. Lastly, coexpression of cortactin and WIP stimulated membrane protrusions.

Conclusions: WIP, a protein involved in filopodia formation, binds to both actin monomers and cortactin. Thus, recruitment of actin monomers to a cortactin-activated Arp2/3 complex likely leads to the observed increase in cortactin activation of Arp2/3 complex by WIP. These data suggest that a cortactin-WIP complex functions in regulating actin-based structures at the cell periphery.

Introduction

Reorganization of the actin cytoskeleton is central to the regulation of cell motility, membrane trafficking, and other cellular events [1]. Extracellular cues that regulate actin dynamics are transmitted from cell surface receptors to Rac and cdc42, two members of the Rho family of small G proteins, resulting in the formation of lamellipodia and filopodia, respectively [2]. Rac and cdc42 mediate signals upstream of various effector proteins, including members of the WASp/SCAR family [3]. WASp/SCAR family members bind and activate Arp2/3 complex via a conserved VCA domain. This domain is composed of one or two verprolin (V) homology domains, which bind actin monomers, a central (C) linking region, and an acidic (A) region, which binds to Arp2/3. Arp2/3 complex mediates the dendritic nucleation of actin filaments [3]. Numerous proteins, such as ActA, WASp/SCAR family, yeast myosin I, cortactin, Abp1p, and yeast Eps15-like endocytic protein, Pan1p, activate Arp2/3 complex [3, 4].

Cortactin was originally identified as an 85 kDa phosphotyrosine-containing protein in cells expressing v-src [5]. Cortactin localizes, in a Rac-dependent manner [6], to the cortical cytoskeleton and lamellipodia [5]. Cortactin is comprised of an N-terminal acidic (NTA) domain followed by 6.5 37-amino acid repeats. Cortactin binding to F-actin is mediated by the amino terminal repeats and requires the fourth repeat [5]. The central region of cortactin includes a proline-rich region that contains three c-src tyrosine phosphorylation sites (Tyr421, 466, and 482) [5]. An SH3 domain resides at the carboxy terminus and mediates interaction with cortBP1, Shank, ZO-1, and dynamin2 [5]. The cortactin NTA domain and the F-actin binding repeat domain are required for Arp2/3 complex activation [7], indicating that the cortactin NTA and repeat domains serve to dock Arp2/3 complex and F-actin, which facilitates actin network formation. Cortactin inhibits actin filament debranching, indicating a role for cortactin in stabilizing cortical actin filaments [7]. Localization of cortactin to the cell periphery coupled with its ability to stimulate and stabilize Arp2/3-dependent actin polymerization in vitro supports a role for cortactin in actin cytoskeletal dynamics [5].

In this study, we identify WIP as a cortactin binding protein and provide evidence that WIP modulates cortactin-mediated Arp2/3 complex activation. WIP was initially identified in a yeast two-hybrid screen as a WASp-interacting protein and displays a wide distribution of expression [8]. Other WIP-interacting proteins include N-WASp [9], profilin [8], and Nck [10]. Overexpression of WIP in lymphoid cells induces actin polymerization and cell surface projections [8]. WIP functions with N-WASp in filopodia formation, implying a role in regulation of actin dynamics [9]. Here, we identify WIP as a cortactin binding protein by using the cortactin SH3 domain as a probe in the yeast two-hybrid assay. We show that the SH3 domain of cortactin interacts with a proline-rich region in WIP. WIP requires the cortactin binding region for efficient localization with cortactin at the cell periphery. WIP increases the efficiency of cortactin-mediated activation of Arp2/3 complex. Lastly, coexpression of cortactin and WIP stimulates membrane protrusions in a manner dependent on an intact cortactin SH3 domain. We conclude that the interaction between cortactin and WIP serves to regulate cortical actin dynamics and lamellipodia protrusion.

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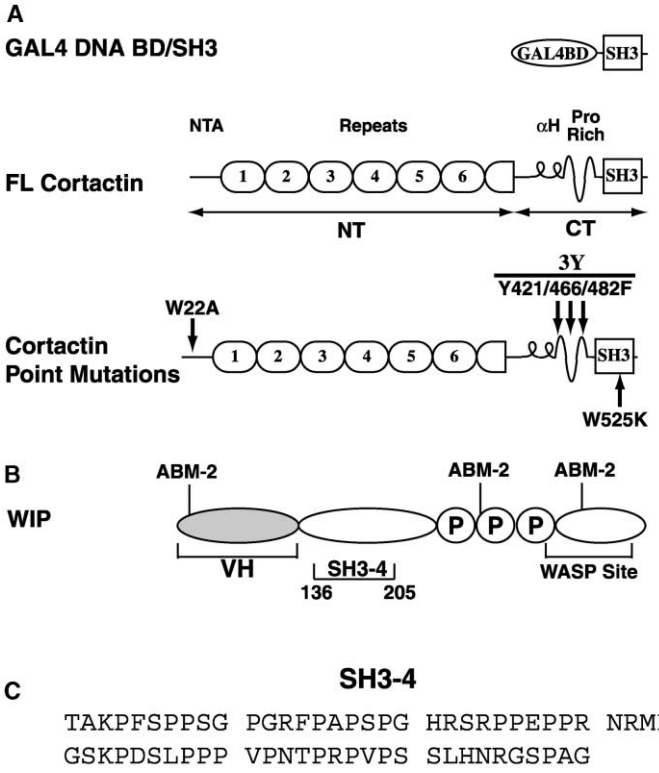

Figure 1. Schematic Representation of Cortactin and WIP Proteins

(A) GAL4 DNA BD/SH3 fusion protein (top) and full-length cortactin (middle). Full-length cortactin is comprised of an N-terminal acidic (NTA) region followed by 6.5 37-amino acid tandem repeats (ovals). A predicted α-helical and proline-rich region is followed by an SH3 domain. The NTA and repeats comprise the amino terminus (NT) of cortactin, while the α-helical, proline-rich region and the SH3 domain comprise the carboxy terminus (CT). Cortactin point mutants (bottom). W22A, tryptophan to alanine point mutation in the NTA domain; 3Y, Y to F point mutations of the three src-phosphorylated tyrosines at 421, 466, and 482; and W525K, tryptophan to lysine point mutation in the SH3 domain.

(B) Schematic diagram of WIP. Identified domains include: VH, verprolin homology; ABM-2, actin-based motility homology-2 (XPPPPP, X is A, S, L, or G); SH3-4, amino acids 136–205, the region of WIP identified from the yeast two-hybrid assay; P, consensus SH3 binding domain (GRSGPXPPXP); WASP interaction site, amino acids 416–488.

(C) The amino acid sequence of the peptide encoded by the SH3-4 cDNA.

Results

The Cortactin SH3 Domain Stably Interacts with WIP

Previously, we used the cortactin SH3 domain fused to the GAL4 DNA binding domain (GAL4 DNA BD/SH3) (Figure 1A, top) in a yeast two-hybrid screen to identify interacting proteins from a rat hippocampal cDNA library [11]. To identify additional cortactin SH3 binding partners, we used a 9.5-day mouse embryo library to conduct a yeast two-hybrid screen. Analysis of approximately 1.9×10^6 colony-forming units yielded 42 clones that were positive in the β-galactosidase selective assay, and this finding is indicative of a potential interaction between the library and probe fusion proteins. Following DNA sequencing, a single clone (SH3-4) was found to encode amino acids 136–205 of WASp-Interacting Protein (WIP) (Figure 1B). The deduced amino acid sequence of clone SH3-4 (Figure 1C) was 94% identical to a region of the rat WIP homolog [12].

To characterize the interaction between WIP and cortactin, glutathione S-transferase (GST) pulldown experiments were performed. Individual GST-cortactin fusion proteins (Figure 1A, bottom) were used to investigate the interaction with ectopically expressed FLAG-WIP in HEK293 cell lysates. The wild-type and C-terminal cortactin fusion proteins, along with the GST-Nck fusion protein, a WIP-interacting protein [10], efficiently bound FLAG-WIP (Figure 2A, top). Other cortactin fusion proteins that demonstrated WIP binding in this assay included GST-FL 3Y and GST-FL W22A (Figure 2A, top). Mutation of the SH3 domain, either in the context of full-length cortactin (GST-FL W525K) or a C-terminal fragment of cortactin (GST-CT W525K) (Figure 1A, bottom), effectively disrupted binding with WIP (Figure 2A, top). Equal amounts of GST-fusion proteins were used in all experiments (Figure 2B).

To determine whether the cortactin and WIP interaction was direct, binding assays were performed with purified 6xHis-tagged WIP (Figure 2C) and purified GST-cortactin. The wild-type and C-terminal cortactin fusion proteins, along with GST-Nck, efficiently bound His-tagged WIP (Figure 2A, bottom). In addition, the cortactin fusion proteins, GST-FL 3Y and GST-FL W22A, efficiently interacted with His-tagged WIP (Figure 2A, bottom). His-tagged WIP failed to bind to cortactin variants containing a mutant SH3 domain (FL W525K and CT W525K) (Figure 2A, bottom).

To measure the apparent binding affinity between cortactin and WIP, increasing amounts of GST full-length cortactin were incubated with a constant amount of purified His-tagged WIP. Based on the data shown in Figure 2D, we estimate an approximate apparent K_d of 0.3 μM (Figure 2D). Together, these data indicate that cortactin directly binds WIP through its SH3 domain.

WIP Associates with Endogenous Cortactin

To assess the interaction of cortactin with WIP in cells, HEK293 cells were transfected with FLAG-WIP or a series of FLAG-WIP deletion mutants (Figure 3A). Wild-type FLAG-WIP was detected in cortactin immune complexes (Figure 3B). In addition, endogenous cortactin bound to FLAG-WIP(112), a WIP variant lacking the first 112 N-terminal amino acids (Figure 3C). Cortactin failed

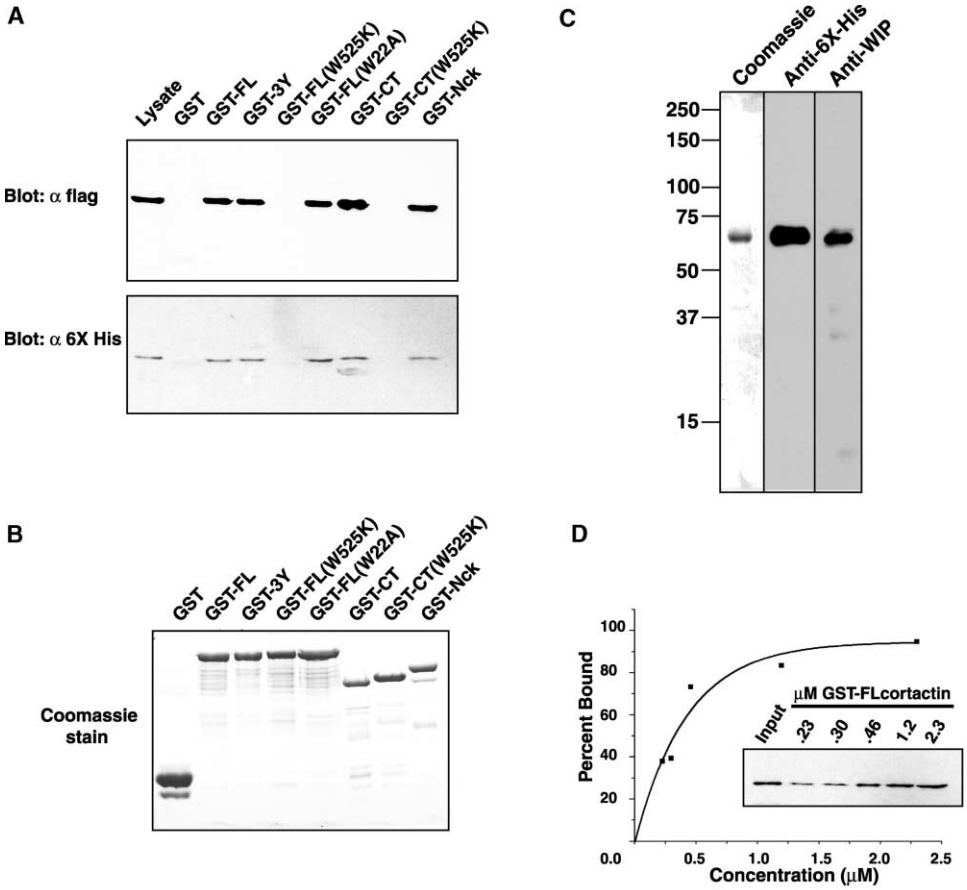

Figure 2. The Cortactin SH3 Domain Interacts with WIP

(A) GST-cortactin interacts with WIP. GST-cortactin pulldown of FLAG-WIP from transfected cell lysates (top) and GST-cortactin pulldown of purified 6xHis-tagged WIP (bottom). Equimolar amounts of GST-cortactin (200 nM/L) or GST-Nck (200 nM/L) fusion proteins were incubated with 600 μg HEK293 cell lysate expressing FLAG-WIP or 25 nM 6xHis-WIP. Following incubation for 30 min, bound complexes were separated by SDS-PAGE and transferred to nitrocellulose, and WIP binding was determined by Western blotting with either M5 anti-FLAG (top) or anti-6xHis (bottom) monoclonal antibodies.

(B) Representative amounts of GST and GST-cortactin fusion proteins were separated by SDS-PAGE and were stained with Coomassie blue.

(C) Purified WIP (12.5 μg) was subjected to 10% SDS-PAGE and was stained with either Coomassie blue (left) or was analyzed by Western blotting with an anti-6xHis monoclonal antibody (1:1000, middle) or an anti-WIP polyclonal antibody (1:10,000, right).

(D) Determination of the apparent binding affinity of cortactin for WIP. Purified 6xHis-WIP (2.1 nM) was incubated with 0.23, 0.30, 0.46, 1.2, and 2.3 μM GST-FL cortactin sepharose for 30 min. The complexes were washed and separated by SDS-PAGE, and bound His-WIP was identified by Western blotting with the anti-6xHis monoclonal antibody. Quantitation was determined by densitometry. The dissociation constant (K_d) for binding was estimated to be 0.3 μM. The inset is a representative Western blot from the assay.

to interact with FLAG-WIP(237), a variant lacking the first 237 N-terminal amino acids, or FLAG-WIP(Δ110–170), a variant containing a deletion of amino acids 110–170 (Figure 3C). Consistent with their inability to interact with cortactin, FLAG-WIP(237) and FLAG-WIP(Δ110–170) lack all or part of the proline-rich region of WIP that defines clone SH3-4 (amino acids 136–205), isolated from the yeast two-hybrid assay. Therefore, these data confirm the interaction of cortactin and WIP in vivo and define the proline-rich region of WIP as the binding region for the cortactin SH3 domain.

Cortactin and WIP Colocalize at the Cell Periphery

In adherent cells, cortactin localizes to cortical actin structures at the cell membrane (e.g., lamellipodia) [5]. In addition, endogenous WIP localizes to filopodia in bradykinin-stimulated cells [9]. To determine whether cortactin and WIP are present in similar subcellular compartments, cells were transfected with wild-type FLAG-WIP or FLAG-WIP variants, and the localization of cortactin and FLAG-WIP was determined by immunostaining. REF52 cells, a cell line used for immunofluorescent analysis of structures at the cell periphery, were grown in serum, and anti-FLAG immunostaining revealed that full-length FLAG-WIP localized to a perinuclear region and to regions of membrane ruffling at the cell periphery (Figure 4A). This localization was coincident with endogenous cortactin staining (Figure 4B), as determined by image merging (Figure 4C). A similar pattern of staining was observed in cells expressing FLAG-WIP(112) (Fig-

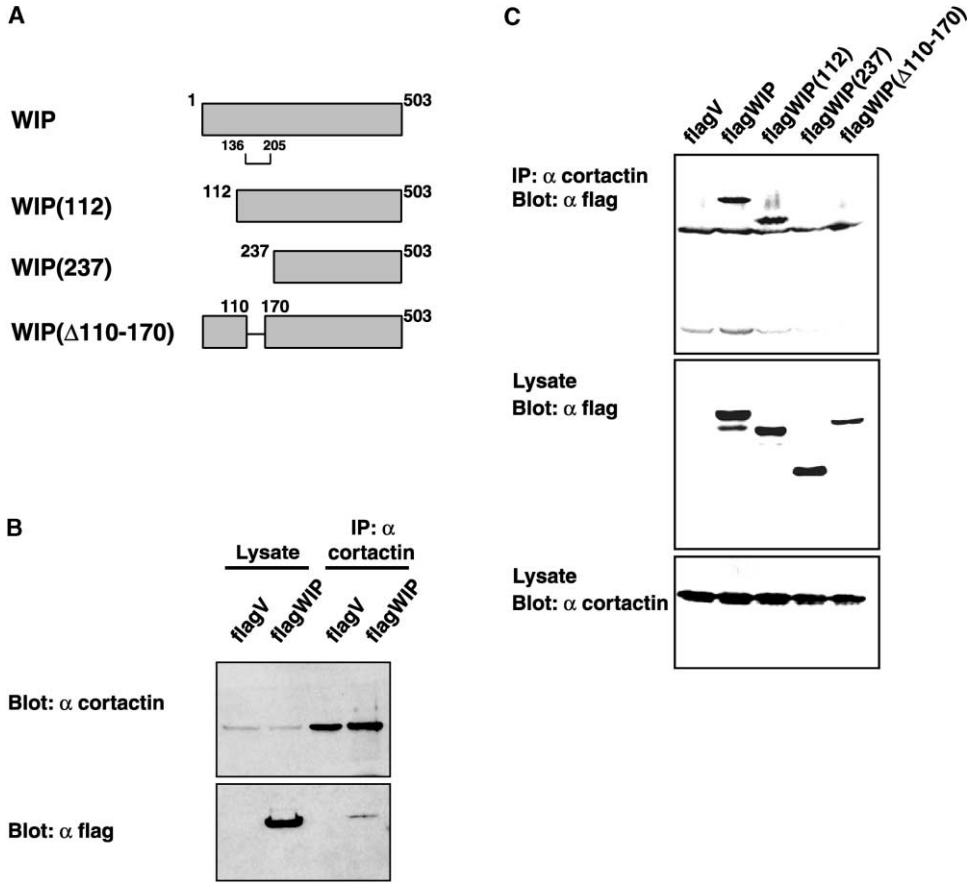

Figure 3. Biochemical Characterization of the Cortactin Binding Region of WIP

(A) A schematic representation of WIP deletion constructs: full-length WIP; WIP(112), deletion of the first 112 amino acids; WIP(237), deletion of the first 237 amino acids; and WIP(Δ110–170), an internal deletion of amino acids 110–170. The bracket denotes the region of WIP (amino acids 136–205) isolated from the yeast two-hybrid screen.

(B) Interaction of WIP with endogenous cortactin. Lysates from HEK293 cells transfected with wild-type WIP were incubated with protein A sepharose beads coupled to the cortactin monoclonal antibody 4F11. Immunoprecipitates were separated by 8% SDS-PAGE and transferred to nitrocellulose, and Western blots were performed with 4F11 (top) and M5 anti-FLAG (bottom) monoclonal antibodies.

(C) Interaction between endogenous cortactin and FLAG-WIP deletion mutants. HEK293 cells were transfected with wild-type FLAG-WIP and the indicated FLAG-WIP deletion mutants, and immunoprecipitation analysis of WIP binding was performed as described in (B).

ures 4D–4F). In contrast, FLAG-WIP(237) and FLAG-WIP(Δ110–170) primarily localized to the perinuclear region, did not display significant immunolocalization at the cell periphery (Figures 4G and 4J), and did not colocalize with cortactin (Figures 4I and 4L). These observations are consistent with the above binding data, suggesting that WIP and cortactin associate at the cell periphery and that the cortactin binding region of WIP is required for efficient cortical localization.

WIP Increases the Efficiency of Cortactin-Mediated Arp2/3 Actin Nucleation

WASp family proteins contain one or more actin monomer binding domains that are required for activation of Arp2/3 complex [3]. In contrast, cortactin is a weak activator of Arp2/3 complex and does not interact with monomeric actin [7, 13]. WIP binds G-actin and is important for N-WASp-mediated Arp2/3 complex activation in cells, but not in vitro [9]. To determine if the interaction of WIP with cortactin influences activation of Arp2/3 complex by cortactin, we monitored the activation of Arp2/3 complex actin nucleation activity by fluorometric measurement of pyrene actin polymerization [14]. The WASp family VCA domain [3] and cortactin [7, 13] independently induce actin polymerization in an Arp2/3 complex-dependent manner (Figure 5A). The addition of substoichiometric amounts of WIP (10 nM) did not affect actin polymerization by Arp2/3 complex and cortactin. However, the addition of increasing concentrations of WIP promoted Arp2/3 complex-mediated actin nucleation, demonstrated by the concentration-dependent increase in the slopes of the actin nucleation curves, which are directly proportional to the concentration of free barbed actin filament ends (Figure 5A). WIP and cortactin alone, without Arp2/3 complex, did not induce actin polymerization. In samples other than those containing wild-type cortactin, Arp2/3 complex, and WIP, WIP had a slight inhibitory effect on actin polymerization, probably due to sequestration of monomeric actin, which prevented spontaneous actin nucleation. Mea-

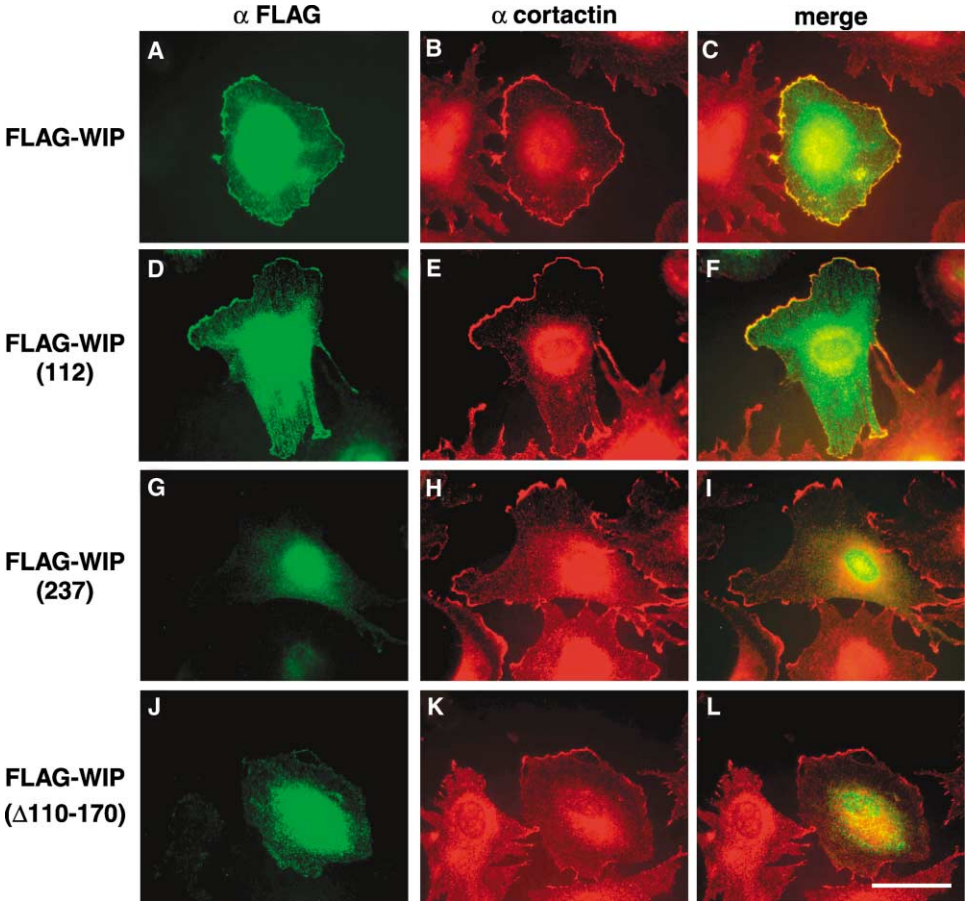

Figure 4. Localization of WIP at the Cell Periphery Requires Amino Acids 110–170

(A–L) REF52 cells were transfected with the FLAG-tagged WIP constructs. Cells were incubated overnight in the presence of 10% FBS, trypsinized, and replated on 1.5 μg/ml fibronectin for 4 hr prior to fixation. (B, E, H, and K) Cells were immunostained for endogenous cortactin by using an anti-cortactin N-term polyclonal antibody (α cortactin). (A, D, G, and J) FLAG-tagged WIP proteins were visualized with the anti-FLAG M5 monoclonal antibody (α FLAG). (C, F, I, and L) Regions of cortactin and FLAG-WIP colocalization were visualized following the pairwise merging of both images. The scale bar represents 50 μm.

surement of the slopes of the pyrene assay curves at increasing concentrations of WIP (50–400 nM) shows a linear increase in the concentration of free barbed ends (Figure 5B).

We next sought to determine whether the increased actin polymerization induced by WIP was dependent on the interaction between cortactin and WIP. The addition of cortactin W525K and Arp2/3 complex induced actin nucleation in a manner similar to that of full-length wild-type cortactin (Figure 5C). However, WIP was not able to accelerate actin nucleation in the presence of cortactin W525K and Arp2/3 complex, and it exhibited less efficient actin polymerization (Figure 5C). Likewise, in the presence of the cortactin W22A mutant, which does not interact with or activate Arp2/3 complex [13, 15], WIP did not accelerate Arp2/3 complex activation. These data indicate that the increased efficiency of actin polymerization observed in the presence of WIP requires the ability of cortactin to interact with both WIP and Arp2/3 complex.

Previous studies with WASp family members show that the addition of actin filaments increases the rate of actin nucleation by Arp2/3 complex [16]. The addition of actin filaments to cortactin and Arp2/3 complex, in the presence or absence of WIP, increases the rate of actin nucleation (Figure 5D). Thus, consistent with data for WASp family proteins, the addition of actin filaments decreases the lag time of cortactin-mediated activation of Arp2/3 complex.

Cortactin inhibits the debranching of actin filament networks in vitro [7]. To determine how WIP affects cortactin-mediated stabilization of actin branches, we performed an in vitro actin debranching assay. Alone, WIP has no effect on actin debranching (Figure 5E). Also, the addition of WIP does not affect the ability of cortactin to stabilize actin branches (Figure 5E). These data indicate that inhibition of actin debranching by cortactin is not influenced by interaction with WIP.

Cortactin and WIP Stimulate Membrane Protrusion

The localization of cortactin to the cell periphery along with its ability to activate the Arp2/3 complex suggests a function for cortactin in regulating cortical actin dynamics. Similarly, WIP affects actin dynamics by mediating filopodia formation downstream of Cdc42 [9]. To

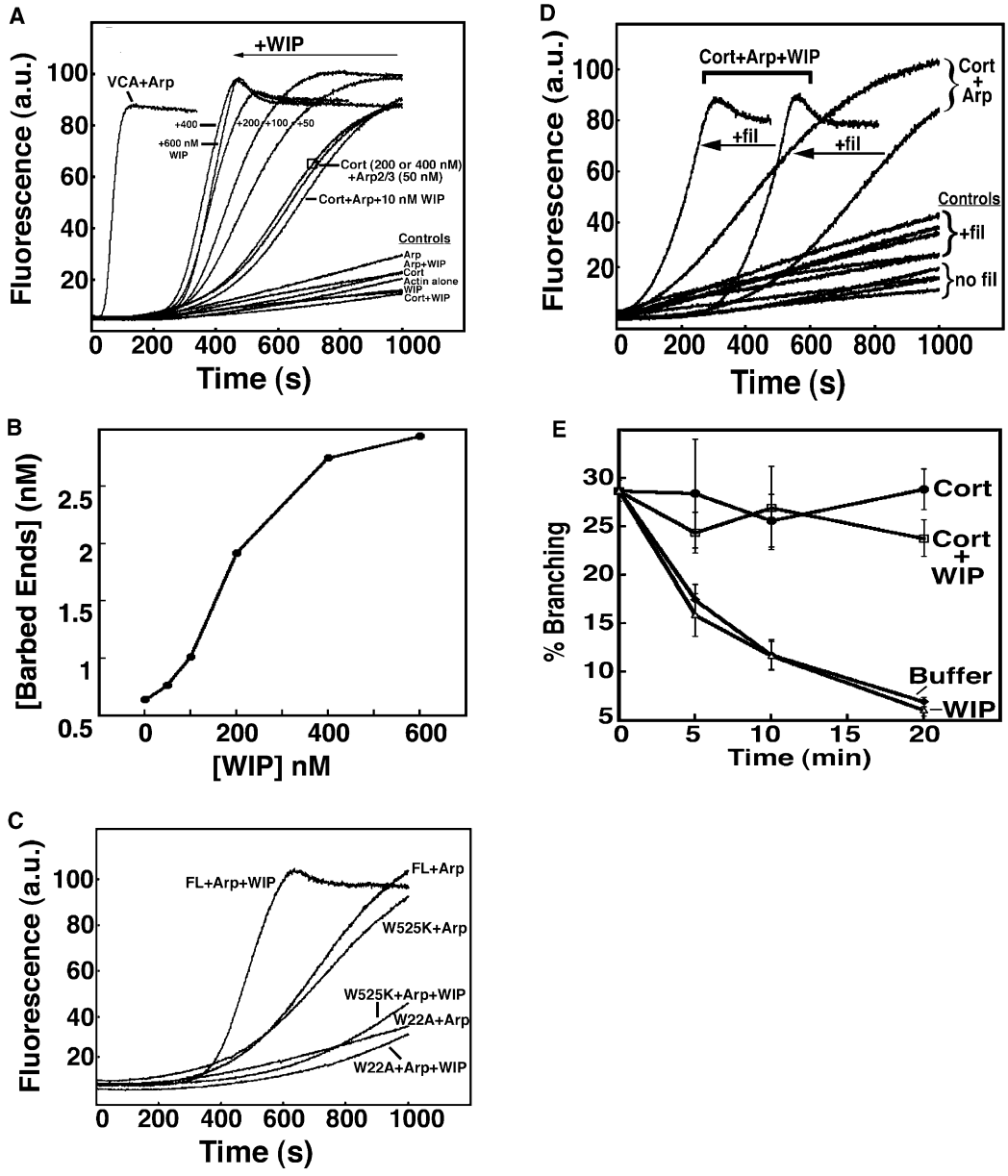

Figure 5. WIP Increases the Efficiency of Cortactin-Stimulated Arp2/3 Complex Activation

The effect of WIP on cortactin-mediated Arp2/3 activation was determined by fluorimetry with pyrene-labeled actin.

(A) Purified Arp2/3 complex was incubated with recombinant wild-type cortactin in the absence or presence of recombinant WIP in polymerization buffer. Controls included a buffer control ("actin"), WIP alone, cortactin alone, Arp2/3 complex alone, cortactin + WIP, and WIP + Arp2/3 complex, as indicated (lower right). The GST-VCA fragment of N-WASp (200 nM) incubated with Arp2/3 complex (50 nM) was a positive control ("VCA + Arp").

(B) Increasing concentrations of WIP (50–400 nM) induce a linear increase in the concentration of free barbed ends. The concentrations of free barbed ends were calculated from the slopes of the curves. The data points represent the average of duplicate determinations.

(C) The ability of WIP to enhance Arp2/3 activation by cortactin requires an intact NTA and SH3 domain. Wild-type ("FL"), W22A and W525K cortactin (200 nM), WIP (400 nM), Arp2/3 complex (50 nM), and monomeric actin (2.5 μM) were coincubated and analyzed by using the pyrene actin polymerization assay as described in (A).

(D) Actin filaments reduce the lag period for Arp2/3 complex activation by cortactin or cortactin + WIP. Arp2/3 complex (50 nM), cortactin (200 nM), WIP (400 nM), and monomeric actin (2.5 μM) were coincubated in the absence or presence (+fil) of 150 nM actin filaments by using the pyrene actin polymerization assay as described in (A). Controls included a buffer control, WIP alone, cortactin alone, Arp2/3 complex alone, cortactin + WIP, and WIP + Arp2/3 complex, in either the absence (no fil) or presence (+fil) of 150 nM actin filaments.

(E) WIP does not affect cortactin-mediated stabilization of actin branches. An actin debranching assay was performed in which monomeric actin (4 μM) was added to the VCA domain (300 nM) and Arp2/3 complex (25 nM) and incubated for 6 min. The samples were incubated with rhodamine-phalloidin (0 min sample), cortactin (200 nM), WIP (400 nM), cortactin and WIP, or buffer. At various time points, samples were applied to glass coverslips, and percent branching was assessed by calculating the percent of actin filaments that were branched at each time point.

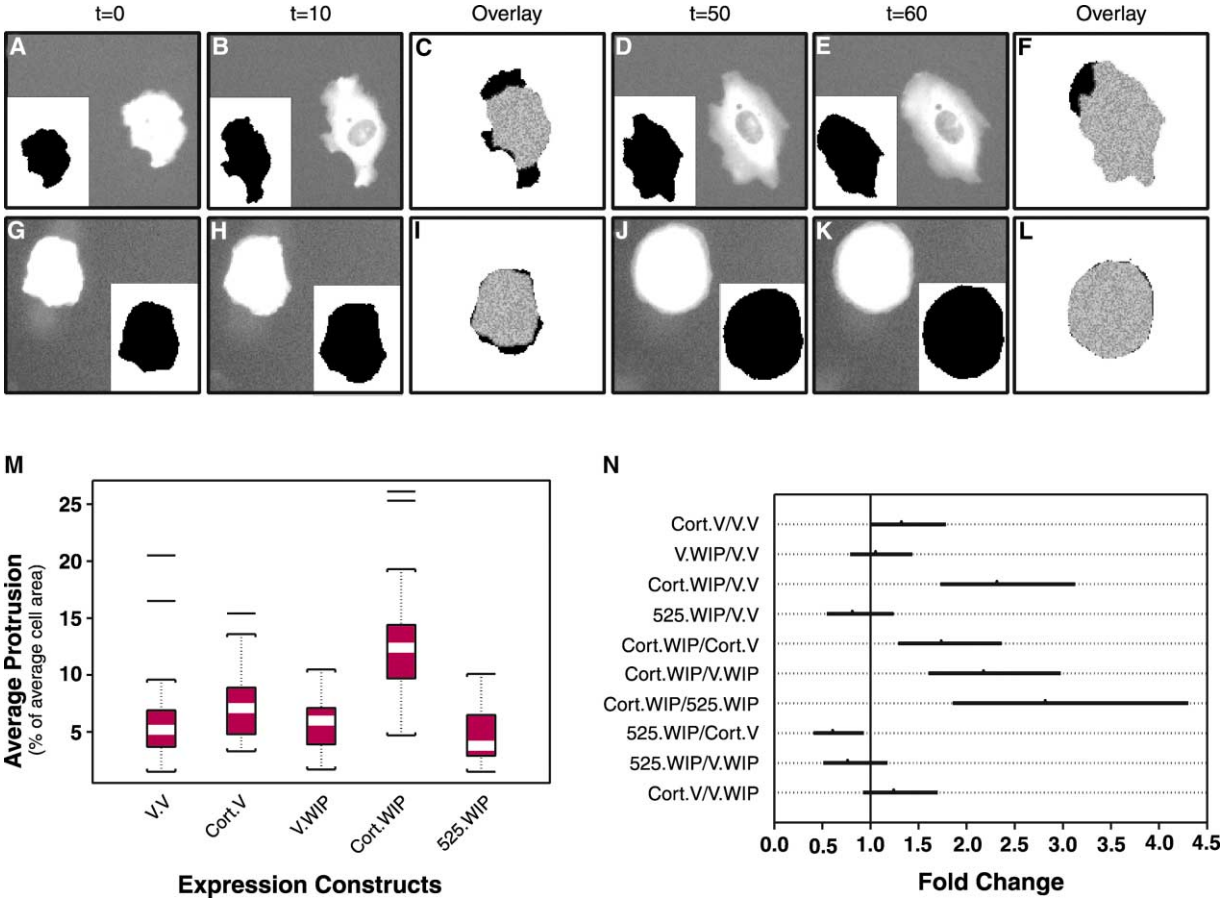

Figure 6. Coexpression of Cortactin and WIP Stimulates Membrane Protrusion

(A–L) Subconfluent CHO K1 cells were cotransfected with expression constructs encoding either GFP-tagged wild-type or W525K cortactin and FLAG-WIP. Cells were trypsinized and replated on fibronectin (1 μg/ml) for 1 hr in CCM1 media. DIC and fluorescent images were acquired every 10 min for a total of 70 min. A representative image sequence of a cell expressing GFP-FL cortactin and FLAG-WIP at times (A) t = 0, (B) t = 10, (D) t = 50, and (E) t = 60 min and a cell expressing the GFP and FLAG vectors at times (G) t = 0, (H) t = 10, (J) t = 50, and (K) t = 60 min are shown. (A, B, D, E, G, H, J, and K) Each frame was converted to a binary image, which is shown in the inset. (C, F, I, and L) Adjacent binary images were overlayed, and the outer black area was quantitated as a positive protrusion for each set of adjacent images.

(M) The average membrane protrusive area for each cell during the 70-min movie is expressed as a ratio of the average cell area in a box plot. Each experimental condition contained between 22 and 25 cells, except for the 525.WIP, which contained 9 cells.

(N) Comparison of the ratio of average protrusions measured with different expression constructs was interpreted as fold change. A 95% confidence interval for fold change that does not contain 1 is considered statistically significant. Exact numerical values are shown in Table 1.

determine whether cortactin and WIP function together in regulating cortical actin cytoskeletal events, we studied the effects of cortactin and WIP on peripheral membrane dynamics by using time-lapse video microscopy. GFP-tagged cortactin, GFP-cortactin W525K, and FLAG-WIP were expressed individually or together, and cell images were acquired at 10-min intervals over a 70-min time span. To ensure that peripheral structures were not excluded from the quantitation, images were taken at longer exposures and the fluorescence image was compared to a simultaneously captured DIC image. Fluorescence images from GFP-cortactin and FLAG-WIP coexpressing cells (Figures 6A, 6B, 6D, and 6E) as well as GFP-vector- and FLAG-vector-expressing cells (Figures 6G, 6H, 6J, and 6K) were converted into binary images (Figures 6A, 6B, 6D, 6E, 6G, 6H, 6J, and 6K inset). Adjacent binary images were overlayed (Figures 6C, 6F, 6I, and 6L). Regions denoting new membrane extensions appear black and were quantitated for each set of adjacent binary images. A box plot depicting the average membrane protrusive area normalized to the area of the cell during the 70-min movie (see the Experimental Procedures) is shown (Figure 6M). Cells expressing GFP-cortactin and FLAG-WIP (Cort.WIP) displayed significantly greater average protrusions than cells expressing GFP- and FLAG-vector (V.V), GFP-cortactin and FLAG-vector (Cort.V), GFP-vector and FLAG-WIP (V.WIP), and GFP-W525K cortactin and FLAG-WIP (525.WIP) (Figure 6N and Table 1). A statistically significant 2.3-fold increase (95% confidence interval, lower 1.7 and upper 3.1) was observed upon expression of cortactin and WIP (Cort.WIP). In addition, coexpression of cortactin and WIP exhibited a statistically significant 2.8-fold increase in average membrane protrusion compared to cells expressing the cortactin SH3 variant (W525K) and WIP. These data are consistent with an

Table 1. Pairwise Comparisons of Variance between the Membrane Protrusions Produced by Using Different Expression Conditions

Construct	Estimate	Lower	Upper
Cort.V/V.V	1.3311	0.9933	1.7839
V.WIP/V.V	1.0634	0.7879	1.4351
Cort.WIP/V.V	2.3245	1.7282	3.1268*
525.WIP/V.V	0.8220	0.5460	1.2375
Cort.WIP/Cort.V	1.7463	1.2898	2.3644*
Cort.WIP/V.WIP	2.1860	1.6044	2.9785*
Cort.WIP/525.WIP	2.8279	1.8578	4.3045*
525.WIP/Cort.V	0.6175	0.4094	0.9315*
525.WIP/V.WIP	0.7730	0.5086	1.1750
Cort.V/V.WIP	1.2518	1.6997	0.9220

A summary of the quantitative pairwise comparison of different variations of expression constructs shown in Figure 5N. A 95% confidence interval not including 1 is considered statistically significant and is denoted with an asterisk.

interaction between cortactin and WIP leading to the increase in membrane protrusions and suggest that cortactin and WIP function together to stimulate dynamic movement of the cortical plasma membrane.

Discussion

Cortactin binds F-actin, localizes to the cell periphery at regions of active actin remodeling, and activates the Arp2/3 complex [5]. These observations are consistent with a role for cortactin in actin cytoskeleton organization. In this study, we have further characterized the role of cortactin in cytoskeleton remodeling by identifying an interaction between cortactin and WIP. The cortactin SH3 domain and a proline-rich region present in WIP mediate the interaction. A variant of full-length WIP lacking amino acids 110–170 does not interact with endogenous cortactin, indicating the importance of this proline-rich domain of WIP for cortactin binding. Using GST-cortactin proteins and purified WIP, we have demonstrated a direct interaction with an approximate apparent K_d of 0.3 μM. Cortactin and WIP display a similar cortical localization at the cell periphery, and recruitment of WIP to the cell cortex requires the cortactin binding domain. WIP increases the efficiency of cortactin-mediated Arp2/3 complex activation, and expression of cortactin and WIP stimulate the formation of membrane protrusions. Together, these data indicate a functional interaction for cortactin and WIP in peripheral actin cytoskeleton remodeling and plasma membrane extension.

Based on the observation that the cortactin SH3 domain interacts with CortBP1, ZO-1, and dynamin2, it has been suggested that cortactin functions to connect regions of dynamic actin assembly to transmembrane receptor complexes and endocytic vesicles [5]. Similar to other cortactin SH3 ligands, WIP localizes with cortactin at the cell periphery; however, unlike other interacting proteins, WIP enhances cortactin-mediated activation of Arp2/3 complex. Thus, cortactin-WIP complexes may serve to act as positive effectors of Arp2/3 complex at sites of actin assembly. It is unclear how cortactin interaction with different ligands is regulated. It is likely that a combination of factors such as subcellular localization or tyrosine and/or serine phosphorylation of either cortactin or its binding partners regulates SH3-dependent binding.

Several studies have indicated a role for WIP in actin dynamics. Recruitment of WIP to vaccinia virus is required for N-WASp localization and actin-based pathogen motility [17]. WIP forms a trimolecular complex with N-WASp and Cdc42 and is required for bradykinin- and Cdc42-mediated filopodia formation [9]. In contrast to the enhancement of cortactin-mediated Arp2/3 complex activation observed after WIP binding, interaction of WIP with N-WASp inhibits Arp2/3 complex activation in vitro [9]. WIP also binds to actin monomers, presumably through a WASp homology 2 (WH2) G-actin binding motif at its amino terminus [9]. Also, WIP binds F-actin and prevents depolymerization of actin filaments [9]. However, WIP did not affect stabilization of actin branches either alone or with cortactin. The association of WIP with the adaptor protein Nck suggests that WIP might be important in transducing signals from receptor tyrosine kinases to the actin cytoskeleton [10]. Thus, interaction with cortactin, a protein proposed to link extracellular receptors to regions of Arp2/3-mediated actin nucleation, might be a key step in mediating WIP function.

Study of the WIP homolog, Vrp1p, in *S. cerevisiae* has provided further insight into the function of WIP. In yeast, Vrp1p localizes to dynamic cortical actin patches analogous to the cortical actin cytoskeleton in mammalian cells [18, 19]. Similar to WIP, Vrp1p contains a WH2 domain [18] and interacts with Bee1/Las17p, the yeast homolog of WASp, through its carboxy terminus [19]. Mutation or deletion of Vrp1p results in observable defects in the actin cytoskeleton and endocytosis [18, 20, 21]. Also, Vrp1p mutant yeast are not viable at 37°C [22]. Expression of mammalian WIP suppresses these defects, which is consistent with the observation that WIP is a functional homolog of Vrp1p [23].

Similar to WIP, Vrp1p has been shown to regulate actin dynamics. Multiple proline-rich domains in Vrp1p interact with the SH3 domain of Myo3p and Myo5p, yeast type I myosins [24]. Both Las17p and type I myosins localize to cortical patches and mediate actin cytoskeletal changes through Arp2/3 complex [25, 26]. The ability to bind and recruit actin monomers is fundamental for the observed activation of Arp2/3 complex displayed by both N-WASp and ActA [27, 28]. In contrast to Las17p, yeast type I myosins do not contain a known actin monomer binding domain, which might explain the observed requirement for Vrp1p in type I myosin-mediated actin polymerization through Arp2/3 complex [29]. By analogy, the studies presented here show that cortactin-WIP interaction enhances actin nucleation through Arp2/3 complex. One possibility is that WIP recruits actin monomers to cortactin-Arp2/3 complexes, thereby enhancing the actin nucleation and polymerization.

WIP inhibits depolymerization of actin filaments [9]. Thus, in addition to enhanced Arp2/3-mediated actin network formation, the concerted effects of a cortactin-WIP complex might be to inhibit depolymerization (WIP) and debranching (cortactin) of resultant Arp2/3-induced actin filament networks.

Conclusions

Using both genetic and biochemical methods, we demonstrate that cortactin, through its SH3 domain, binds to a proline-rich region of WIP. In cells, cortactin and WIP colocalize in the cytoplasm and at the cell periphery. Using a kinetic assay of actin polymerization, we show that interaction with WIP enhanced cortactin-mediated activation of Arp2/3 complex. Lastly, coexpression of cortactin and WIP stimulate the formation of membrane protrusions. We conclude that cortactin and WIP interaction positively regulates actin network structure at the cell periphery.

Experimental Procedures

DNA Constructs

The pGBT10-SH3 construct was produced by PCR amplification of the murine cortactin SH3 domain and was subcloned into the pGBT10 vector. A GFP-cortactin expression construct was created by PCR amplification of murine cortactin and was subcloned into pEGFP-C1 (Clontech). For construction of the GFP-cortactin (W525K), full-length FLAG-tagged cortactin (W525K) [11] was subcloned into pEGFP-C1. For construction of the GST-cortactin prokaryotic expression cDNAs, pRK5myc-FL, pRK5myc-FL W525K, and pRK5myc-C-term were subcloned into pGST-parallel 2. The GST-FL W22A construct was produced from the GST full-length construct by using the QuikChange site-directed mutagenesis kit (Stratagene). The GST-CT W525K construct was described previously [11]. Using site-directed mutagenesis, the GST-FL 3Y construct was produced from a FLAG-tagged construct encoding wild-type murine cortactin [30]. The GST-Nck construct and the FLAG-tagged WIP constructs were gifts from K. Ravichandran (University of Virginia) [31] and D. Billadeau (Mayo Clinic, Rochester, MN) [32], respectively.

Yeast Two-Hybrid Analysis

The yeast transformation and the β-galactosidase assay was performed as described previously [33, 34]. Positive library plasmids were sequenced and compared to existing databases by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Antibodies and Western Blotting

The cortactin antibodies 4F11 and anti-C-term have been described previously [6, 35]. The following antibodies were purchased: M5 antibody against the FLAG epitope (Sigma-Aldrich), anti-6xHis monoclonal antibody (Clontech), and horseradish peroxidase-coupled secondary antibody (Amersham Biosciences). The Western blotting procedure was described previously [36].

Cell Culture and Transfection

REF52, HEK293 cells, and CHO K1 cells were grown in D-MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were transfected with PolyFect (Qiagen) according to the manufacturer's protocols.

Immunofluoresence Microscopy

REF52 cells were transfected with FLAG-tagged WIP mutants, cultured overnight, and plated on fibronectin (1.5 μg/ml)-coated coverslips. The cells were fixed, immunostained, and imaged as described previously [6].

Protein Preparation and Purification

Arp2/3 complex was purified from bovine thymus by conventional chromatography [37]. GST-VCA was purified by affinity chromatography [38]. Actin was purified from chicken pectoralis skeletal muscle [39] and was gel filtered [40]. GST-cortactin fusion proteins were purified from *E. coli* by glutathione affinity chromatography as previously described [30]. Cortactin proteins were purified by anion exchange chromatography on a Mono-Q column (Pharmacia).

WIP-expressing baculovirus was prepared by PCR amplification from the FLAG-WIP expression construct [32]. The amplified WIP cDNA was subcloned into the baculovirus transfer vector pAcHLT-B (Pharmingen) and was cotransfected with wild-type baculoviral DNA into Sf9 cells. GST-cortactin variants and GST-Nck proteins were purified as described previously [11].

Binding Assays

For immunoprecipitation experiments, cell lysate from HEK293 cells transfected with FLAG-tagged WIP constructs was incubated with 4F11 coupled to Sepharose A resin (Sigma) for 2 hr at 4°C. GST fusion protein pulldown experiments and in vitro binding assays were performed as described previously [11, 30].

Actin Polymerization and Debranching Assays

Pyrene actin polymerization and actin debranching assays were performed as described [7, 14].

Membrane Protrusion Assay

Subconfluent CHO K1 cells were transfected with GFP-cortactin and FLAG-tagged WIP constructs, incubated overnight, and replated on 1 μg/ml fibronectin (Sigma). DIC and fluorescent images were recorded at 10-min intervals for 70 min. Binary images were generated, adjacent binary images were overlayed, and a new protrusive area was quantitated. For each cell, values for average protrusion per 10 min were expressed as a ratio of the cell's average area during the 70-min time span.

Supplementary Material

Supplementary Material including additional details regarding the methods used is available at http://images.cellpress.com/supmat/supmatin.htm.

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